Effect of genetic background and post-infectious stress on visceral sensitivity in Citrobacter rodentium infected mice
Running title: post-infectious visceral hypersensitivity

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ABSTRACT

Background: Infectious gastroenteritis is a major risk factor to develop post-infectious irritable bowel syndrome (PI-IBS). It remains unknown why only a subgroup of infected individuals develops PI-IBS. We hypothesize that immunogenetic predisposition is an important risk factor. Hence, we studied the effect of *Citrobacter rodentium*-infection on visceral sensitivity in Th1-predominant C57BL/6 and Th2-predominant Balb/c mice.

Methods: Eight weeks old mice were gavaged with *Citrobacter rodentium,* followed by 1 hour of water-avoidance stress (WAS) at 5 weeks PI. At 10, 14 days and 5 weeks PI, samples were assessed for histology and inflammatory gene expression by RT-qPCR. Visceral sensitivity was evaluated by visceromotor-response-recordings (VMR) to colorectal-distension.

Key results: *Citrobacter rodentium* evoked a comparable colonic inflammatory response at 14 days PI characterized by increased crypt length and upregulation of Th1/Th17 cytokine mRNA levels ($p_{uncorrected}<0.05$) in both C57BL/6 and Balb/c mice. At 5 weeks PI, inflammatory gene mRNA levels returned to baseline in both strains. The VMR was maximal at 14 days PI in C57BL/6 (150±47%;p=0.02) and Balb/c mice (243±52%;p=0.03). At 3 weeks PI, the VMR remained increased in Balb/c (176±23%; p=0.02), but returned to baseline in C57BL/6 mice. At 5 weeks PI, WAS could not re-introduce visceral hypersensitivity (VHS).

Conclusions&Inferences: *Citrobacter rodentium* infection induces transient VHS in C57BL/6 and Balb/c mice, which ~~is more pronounced and~~ persisted one week longer in Balb/c mice~~, suggesting that a Th2 background may represent a risk factor for prolonged PI-VHS~~. Although other strain-related differences may contribute, a Th2 background may

represent a risk factor for prolonged PI-VHS. As PI-VHS is ~~only~~ transient, other factors ~~seem~~ are crucial for persistent VHS development as observed in PI-IBS.

Key words: Irritable Bowel Syndrome, visceral hypersensitivity, citrobacter rodentium

KEY MESSAGES

General statement: Visceral hypersensitivity (VHS) is a hallmark of (post-infectious) irritable bowel syndrome (PI-IBS) but the underlying mechanisms remain largely unknown.

Aims/goals: We studied whether immunogenetic background and acute stress in the post-*Citrobacter rodentium* infectious phase influence the development of VHS.

Basic methodology: Visceral nociception was assessed by visceromotor-response-recordings (VMR) to colorectal distension. Colonic inflammation was evaluated by RT-qPCR and H&E staining.

Summary: In the acute infectious phase, *Citrobacter rodentium* evoked maximal visceral pain perception in Th1 and Th2 predominant C57BL/6 and Balb/c mice respectively. Visceral nociception remained increased in Balb/c but not in C57BL/6 mice at 3 weeks PI. Five weeks PI, inflammation was completely resolved and VMR returned to normal in both strains. Acute water avoidance stress could not re-introduce VHS, regardless of the immunogenetic background.

INTRODUCTION

Three to 31 % of individuals develop irritable bowel syndrome (IBS)[1] following an infectious gastroenteritis[2-11] and are referred to as post-infectious IBS patients (PI-IBS). Symptoms vary from patient to patient but typically include chronic abdominal pain, bloating and altered defecation patterns in the absence of an organic cause[12]. Visceral hypersensitivity, defined as increased sensitivity to visceral stimuli such as luminal distension, is one of the hallmarks of IBS[13] and can persist for years after the initial infection[14]. Up to date, it is unknown why only a subgroup of infected individuals will develop PI-IBS.

The risk to develop PI-IBS varies with the infectious agent[15-18] with *Campylobacter jejuni*, *Salmonella*, *Shigella* and *Escherichia coli* as main pathogens. Human *Escherichia coli* colitis can be modeled by the *Citrobacter rodentium* murine model of self-limiting colitis, as *C. rodentium* shares 67% of its genes with the human enteropathogenic and enterohaemorrhagic *E. coli*[19, 20]. Based on these observations, *C. rodentium* infection may represent a potential model of PI-IBS. Previously, Ibeakanma C. et al. showed that *C. rodentium* infection in the Th1 predominant mouse strain C57BL/6 mice, evoked hyperexcitability of colonic dorsal root ganglia (DRG) neurons and increased afferent nerve firing that persisted until 30 days PI[21]. Stress concurrently with the infection enhanced neuronal excitability, while repeated water avoidance stress in the PI phase produced no greater enhancement than stress applied alone[21, 22], indicating that stress at the time of infection seems to increase the risk to develop post-infectious visceral hypersensitivity.

Microscopic inflammation has been well documented in PI-IBS and is believed to underlie PI-IBS pathophysiology[2, 23-25]. Serial rectal biopsies taken from patients who developed IBS after Campylobacter *jejuni* gastroenteritis showed a persistent inflammatory infiltrate, with an increase in enterochromaffin cells and T lymphocyte cell counts[2]. Increased mast cell

numbers have been observed in PI-IBS[24] and non PI-IBS[26], showing significant correlations between IBS severity and mast cell counts, spontaneous mast cell tryptase release and colonic permeability[26]. Additional evidence includes upregulation of the pro-inflammatory cytokine interleukin-1β (IL-1β) in rectal biopsies of PI-IBS patients[27], and increased pro-inflammatory cytokine release, f.e. TNF-α, IL-1β and IL-6, by peripheral blood mononuclear cells[28]. Based on the knowledge that mast cells play a key role in IBS and are classically involved in a Th2 immune response, we speculate that the immunogenetic background of the host may be an important component dictating the nature of the immune response and the subsequent development of PI-IBS.

To gain insight into the role of immunogenetic background as a risk to develop VHS after an infection, we assessed the course of infection and visceral sensitivity in *C. rodentium* infected Th1 predominant C57BL/6 and Th2 predominant Balb/c mice. Moreover, we assessed if stress in the PI phase can re-initiate visceral hypersensitivity.

MATERIALS AND METHODS

All experiments were conducted in accordance with the institutional guidelines and approved by the animal ethical committee of the KU Leuven (protocol numbers P179/2009 and P109/2010).

Animals

Six weeks old male C57BL/6 and Balb/c mice were purchased from Janvier (Saint-Berthevin Cedex, France) and left undisturbed for at least 1 week for acclimatization. Animals were maintained under a 14:10h dark/light cycle, at a temperature of 20-22 °C, provided with food and water *ad libitum*.

Citrobacter rodentium (C. rodentium) infection

C. rodentium (DBS100, ATCC® 51459™, Teddington, United Kingdom) was cultured overnight in Luria-Bertani broth (LB) medium (MP Biomedicals, Drogenbos, Belgium) (37 °C, 200 rpm). Fifteen hours later, the bacteria were centrifuged (4 °C, 2000 rpm, 10 minutes) and fresh LB medium was added to dissolve the pellet. Eight weeks old mice were infected by oral gavage of 3 x 10^{10} colony forming units *C. rodentium* or sterile 0.9 % NaCl (B. Braun Medical NV/SA, Diegem, Belgium) followed by intraperitoneal (i.p.) injection of 200 µl sterile 0.9 % NaCl to prevent dehydration[29, 30].

Water avoidance stress (WAS) model

At 5 weeks post-infection, a bucket with a platform of 40 mm diameter was filled with fresh room temperature water (20 °C) up to 1 cm of the top of the platform. Mice were placed on the platform for 1 hour and the visceromotor response was measured 24 hours later[31].

Telemetric Visceromotor Response (VMR) recordings

Mice of at least 20 grams were implanted with Physiotel ETA-F10 telemetric transmitters (Data Sciences International, MC s'Hertogenbosch, The Netherlands). Hereto, mice were anaesthetized by i.p. injection of 20 mg/kg ketamine (Nimatek, Eurovet Animal Health B.V., AE Bladel, The Netherlands) and 1 mg/kg xylazine (Rompun 2 %, Bayer, Diegem, Belgium) and placed on a heating pad (± 30 °C). The telemetric transmitter was inserted in the abdominal cavity, the electrodes were tunneled through the abdominal wall using a 18G needle (Terumo Europe n.v., Leuven, Belgium) and the non-insulated tips were sutured in parallel (± 5 mm apart) into the left external abdominal oblique muscle. After surgery, mice recovered for 12 hours on a heating pad where after they were left undisturbed for 10

consecutive days[32]. The radio telemetry experimental setup for measurement of the visceromotor response (VMR) to colorectal distension (CRD) in mice was adapted from[33, 34].

Colorectal distensions were performed to evoke abdominal cramping as read-out of visceral nociception. Hereto, a distension catheter (Fogarty catheter for arterial embolectomy, 4F; Edwards Lifesciences, Breda, The Netherlands) was inserted into the colon (3 cm from the rectum) of conscious, non-restraint, mice and distended with volumes progressively increasing from 20 µL to 80 µL, with each step lasting 10 sec and separated by 5 min non-distension periods in-between[35]. The VMR responses were measured and quantified using Acknowledge 3.2.6 software (BIOPAC Systems Inc., Goleta, California, USA). For analysis, the mean value of the resting EMG signal 10 sec prior to distension (i.e. basal activity) was subtracted from the mean value of the electromyography signal evoked during the 10 sec distension. Data are presented as % VMR response ± SEM relative to maximum nociception response before infection (i.e. 80 µl distension is set at 100 %) or as area under the curve of the VMR responses.

Evaluation of colonic inflammation by real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 30-50 mg intestinal tissue using the RNeasy Minikit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. cDNA of 2 µg total RNA was transcribed by the qScript cDNA supermix (Quanta Biosciences, Gaithersburg, Maryland, USA) according to manufacturers' instructions.

The primer sequences used to quantify inflammatory gene mRNA are listed in Table 1. Ten µl reaction mix per well was loaded onto a LightCycler® 480 multiwell plate 96 (Roche GmBH, Mannheim, Germany) containing 2.5 µl of each cDNA sample together with 5 µl FastStart

Essential DNA Green Master (Roche GmBH, Mannheim, Germany), 0.2 µl oligonucleotides (10 µM) and 2.3 µl RNase Free Water (Applied Biosystems, Halle, Belgium). Gene expression was normalized to an endogenous reference gene, β-actin. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ [36] and data are presented as relative expression ± standard error of the mean (SEM).

Histopathology assessment

At 10, 14 days and 5 weeks post-infection, full thickness intestinal tissue samples were freshly frozen in Tissue-Tek O.C.T.™ compound (Sakura Finetek Europe B.V., AV Alphen aan den Rijn, The Netherlands). Eight µm cryosections were cut and stained with heamatoxylin & eosin (H&E). Slides were reviewed using an Olympus BX41 light microscope (Aartselaar, Belgium) and scored blinded. H&E staining was performed to assess the degree of colitis using crypt length, crypt space and muscle thickness to address epithelial changes and overall mucosal architecture of each specimen[37]. All measurements were performed using Cell^F software (Aartselaar, Belgium). Mean crypt height was calculated from 5-10 individual measurements of the lengths of all well oriented crypts on each specimen slide.

Statistical analysis

All statistical analyses were performed with Graphpad Prism software (GraphPad Software, San Diego, California, USA). Non-infected versus *C. rodentium* infected groups were compared by Unpaired t-test with Welch's correction. Results are presented after Bonferroni correction for multiple testing (for RT-qPCR data based on 13 genes, $p_{uncorrected}<0.004$ was considered to be significant). VMR recordings were analyzed by 2-way ANOVA with

Bonferroni post-hoc correction. A p value < 0.05 was considered significant. Data are presented as mean + SEM.

RESULTS

C. rodentium infection evokes transient colonic inflammation in C57BL/6 and Balb/c mice

All animals were monitored daily and the majority of animals (85–90%) exhibited signs of illness (e.g. decreased activity) for 2–3 days following infection but recovered quickly. None of the animals died. Infected C57BL/6 mice lost 5% of their initial body weight compared to 2% in Balb/c mice within the first 4 days post-infection (PI). Up to 2 weeks PI, infected C57BL/6 (Fig. 1A) and Balb/c (Fig. 2A) did not reach the body weight of the non-infected controls.

To identify potential differences in the degree of inflammation evoked by *C. rodentium*, inflammatory marker mRNA expression and histology were assessed in colon and small intestine at 10 and 14 days PI. Small intestinal *IFN-γ*, *TNF-α* and *IL-1β* mRNA expression was increased in *C. rodentium* infected C57BL/6 mice at 10 days PI but this increase was not significant after correction for multiple testing (Supplementary Fig. 1A). In contrast, *MCP-1* mRNA levels were significantly decreased (p unpaired t-test Welch's correction = 0.0001) at 10 days PI in the small intestine of infected Balb/c mice (Supplementary Fig. 1B).

At 10 days PI, colonic *c-kit* mRNA expression was significantly decreased in infected C57BL/6 mice compared to non-infected controls (p unpaired t-test Welch's correction = 0.0037; Fig. 1B). *MCP-1*–and *IL-10* mRNA expression was increased in infected C57BL/6 mice, but did not remain significant after correction for multiple testing (Fig. 1B). At 14 days PI, there was a tendency towards increased *IL-1β, IL-17* and *IL-10* mRNA expression while *c-*

kit mRNA expression was decreased in infected C57BL/6 mice, these results did not remain significant after correction for multiple testing (Fig. 1B).

In contrast, infected Balb/c mice showed decreased colonic mRNA levels of *MCP-1* and increased *IL-10* mRNA expression compared to non-infected controls at 10 days PI (p unpaired t-test Welch's correction < 0.004; Fig. 2B). *IFN-γ, TNF-α*, *IL-1β* and *IL-17* mRNA were also upregulated, but could not withstand correction for multiple testing. At 14 days PI, *TNF-α, IL-17* and *IL-10* mRNA levels were increased (p unpaired t-test Welch's correction <0.004, Fig. 2B). Of note, colonic mast cell *tryptase a/b* mRNA expression was increased at 10 and 14 days PI in infected Balb/c mice, but could not withstand correction for multiple testing (Fig. 2B).

Comparison of colonic inflammatory gene expression between infected C57BL/6 and Balb/c mice in the acute inflammatory phase revealed only increased *tryptase a/b* levels in Balb/c mice (unpaired t-test Welch's correction p = 0.004) (Supplementary Fig. 2) and a tendency for increased *IL-17* mRNA and decreased *IL-6* in Balb/c mice, but this could not withstand correction for multiple testing. At 5 weeks PI, *IFN-γ* and *IL-10* mRNA levels were significantly lower in infected Balb/c compared to infected C57BL/6 mice (Supplementary Fig. 2).

H&E staining was performed to assess the degree of colitis using crypt length, crypt space and muscle thickness to address epithelial changes and overall mucosal architecture. Histology of the colon at 10 days PI shows signs of colitis as reflected by increased crypt length in both infected C57BL/6 (Fig. 3A) and Balb/c (Fig. 3B) mice compared to non-infected controls (p unpaired t-test Welch's correction = 0.029 and p <0.0001, respectively), an effect that was more pronounced at PI day 14 (Fig. 3A, B). No changes in muscle thickness or space between the crypts were observed between non-infected and infected mice,

irrespective of genetic immune background (data not shown). Based on the inflammatory cell infiltrate in mucosa and submucosa, mild hyperplasia and minimal goblet cell loss, we can conclude that *C. rodentium* induces a mild colonic inflammation in both mouse strains.

At 5 weeks PI, in the absence of histological changes (Fig. 3A, B), infected C57BL/6 but not Balb/c mice still showed a tendency, albeit not statistically significant after multiple testing correction, towards increased colonic inflammatory mRNA expression of *IL-1β, IL-6, IFN-γ* and *IL-17* compared to non-infected controls (Fig. 2B).

Taken together, *C. rodentium* evokes a transient mild inflammatory response characterized by upregulation of inflammatory cytokine mRNA in both mouse strains at 10 and 14 days PI, with pronounced *IL-17* mRNA upregulation.

C. rodentium infection induces transient VHS in C57BL/6 and Balb/c mice

Colorectal sensitivity was assessed by VMR at 2, 3 and 4 weeks PI and 24 hours post-WAS at 5 weeks PI. At 2 weeks PI, in the presence of colonic inflammation, visceral sensitivity was significantly increased compared to baseline in both infected C57BL/6 (Fig. 4A) and Balb/c (Fig. 4B) mice. The response to colorectal distension (increase in visceral nociception at the maximum distension volume compared to pre-infection) was increased by 50 + 16% in C57BL/6 (p 2-wayANOVA=0.02) and by 143 + 31% in Balb/c (p 2-wayANOVA=0.03) mice. At 3 weeks PI, visceral sensitivity to colorectal distension returned to baseline levels in C57BL/6 mice, while the VMR response to colorectal distension was still significantly increased by 76 + 14% in Balb/c mice (p 2-wayANOVA=0.02). Only Balb/c mice with the highest VMR response at 2 weeks PI remained, to a lower extent, hypersensitive at 3 weeks PI (Supplementary Fig. 3). Visceral sensitivity normalized at 4 weeks PI in both mouse strains (Fig 4 A-D).

As stress may exacerbate or re-initiate visceral sensitivity[22, 38, 39], we next assessed the effect of acute water avoidance stress (WAS) on post-infectious visceral sensitivity. One hour of WAS did not re-install VHS at 5 weeks PI (Fig. 4A-D). Visceral sensitivity to colorectal distension was similar to that before the infection, for both mouse strains (Fig. 4A-D).

DISCUSSION

In humans, it remains unclear why only a subgroup of infected individuals develops long-term PI-IBS. Based on the importance of mast cells in IBS symptom generation[25, 40-42], we hypothesized that a Th2 immune background may increase the risk to develop PI-IBS and used *Citrobacter rodentium* as a murine model to study our hypothesis. In the present study, we evaluated the role of *C. rodentium*-induced inflammation and acute stress on visceral sensory function in Th1-predominant C57BL/6 and Th2-predominant Balb/c mice. *C. rodentium* induced a self-limiting colitis in both strains with induction of colonic inflammation and increased visceral sensitivity to colorectal distension at 2 weeks post-infection. The increase in visceral nociception was transient and lasted 1 week longer in the Th2-predominant Balb/c mice. An episode of acute water avoidance stress (WAS) did not re-initiate PI-VHS irrespective of the immunogenetic background. These results suggest that a Th2-predominant immunogenetic background may represent one of the risk factors to develop prolonged abnormal visceral nociception following an episode of infectious gastroenteritis. ~~This VHS is however transient, suggesting that other factors or triggers, resulting in a sustained abnormal pain response as observed in PI-IBS patients, must be involved.~~ Of note, other strain-related factors, such as differences in nociception and behavior, may undoubtedly contribute as well.

In mice, *C. rodentium* is known to induce an acute, self-limiting colitis, histologically associated with crypt hyperplasia and goblet cell depletion[43, 44]. The infection serves as a

model for human infectious gastroenteritis induced by enteropathogenic *Escherichia coli*, a well-known trigger for PI-IBS in humans[45]. Upon infection, *C. rodentium* transiently colonizes the distal colon with a peak of infection around 10 – 14 days PI in both C57BL/6[21] and Balb/c mice[46]. In line, we showed that *C. rodentium* induced a transient colonic inflammation, characterized by increased cytokine mRNA levels, irrespective of the genetic background, with overt inflammation at 14 days post-infection. The more pronounced colonic expression of *IFN-γ*/*IL-17*, identified as crucial players for host defense against infection[47] at day 14 PI indicates that the peak of infection for both Th1 and Th2 predominant mice lays around 14 days post-infection. Inflammatory cytokine mRNA expression was however not significantly different between the two mouse strains, except for increased *tryptase a/b* mRNA levels in infected Balb/c mice compared to infected C57BL/6 mice. The peak of infection/inflammation was associated with an increase in visceral nociception to colorectal distension in both strains at 2 weeks PI. The duration of increased nociception differed however, i.e. Balb/c mice showed increased VMR responses to colorectal distension up to 3 weeks PI (76% increase) while visceral perception of C57BL/6 mice was already normalized.

Of interest, we noticed that mainly mice with a very high VMR response at 2 weeks PI (in particular Balb/c mice) remained VHS at 3 weeks PI (Supplementary Fig. 3), indicating that the magnitude of the VMR response at 2 weeks PI may be associated with a slower recovery and increased duration of the aberrant nociceptive response. One potential explanation may be the difference in immunogenetic background leading to more pronounced mast cell activation in the Th2 prone Balb/c mice, as suggested by increased *tryptase a/b* mRNA expression in the acute infectious phase in Balb/c mice compared to infected C57BL/6 mice. However, the degree of upregulation was relatively small questioning its physiological relevance. Moreover, Th2-associated cytokine expression did not differ between *C. rodentium* infected

C57BL/6 and Balb/c mice, suggesting that other factors contribute to the prolonged VHS observed in infected Balb/c mice. In fact, there are indeed documented strain differences with respect to behavioral[48, 49] and nociceptive tests[50, 51], most likely contributing to the prolonged VHS in Balb/c mice. It should also be emphasized though that the VHS observed in both strains completely normalized during the PI phase (i.e. at 4 weeks PI), while patients with PI-IBS continue to have symptoms for several years following the infectious episode. These findings are in accordance with other studies, showing no increased VMR response in C57BL/6 mice 30 days PI[22]. Hence, other mechanisms may be critical for the development of chronic VHS.

Psychological comorbidities such as stress, anxiety, depression and adverse early-life events are known to induce and/or exacerbate IBS symptoms[52-57]. Previously, van den Wijngaard et al.[58] showed long-lasting VHS in response to an episode of acute stress, induced by water-avoidance stress (WAS), in a rat model of maternal separation[58, 59]. Therefore, we evaluated if a previous gastrointestinal infection would increase the risk to develop VHS in response to WAS. In the present study however, acute WAS at 5 weeks PI did not recreate VHS, irrespective of genetic background. Our data therefore seem to indicate that stress applied after an intestinal infection is not a major trigger to develop long-lasting VHS. Indeed, Spreadbury et al.[60] showed that chronic psychological stress after clearance of the infection did not have an additional effect on neuronal excitability compared to non-infected mice exposed to the same stressor[60]. Nevertheless, these data confirm that the bacterial infection *per se* does not alter visceral perception. It should be stressed though that psychological co-morbidity *prior* to and not after clearance of the infection is associated with an increased risk to develop IBS[22, 60, 61]. In line, stress during or before *C. rodentium* infection results in an exaggerated peripheral nociceptive signaling compared to *C. rodentium* alone[22] and to

enhanced excitability of dorsal root ganglion neurons compared to non-infected controls[60]. Altogether, these data highlight the importance of the timing of stress relative to the infection. This was indeed further corroborated by our recent study showing that psychological comorbidity prior to or during a gastrointestinal infection predisposes individuals to develop IBS. Of note, we showed that the type of immune response raised against the infection is associated with the risk to develop IBS. Patients who developed a Th2-predominant cytokine profile at the time of infection had an increased risk of PI-IBS 1 year later[62]. These data together with our current findings seem to support the hypothesis that the immunogenetic background may, at least to some extent, contribute to the risk to develop PI-IBS. Nevertheless, the duration of VHS in our murine PI model is rather short lasting, so clearly other factors must be involved.

Of interest, recent evidence suggests that food allergens may be involved in IBS. Not only do more than 60% of patients with IBS report the onset or worsening of symptoms after meals[63], submucosal instillation of food antigens in the duodenum was recently shown to evoke a local reaction with an instant influx of inflammatory cells and increased secretion[64]. Although the exact mechanism underlying these phenomena remains to be determined, one may speculate that an aberrant immune response to food antigens could be involved. Currently, we are investigating the hypothesis that prolonged VHS following a gastrointestinal infection may result from recurrent mast cell activation due to an aberrant immune response mounted against harmless intraluminal antigens present at the time of infection. If true, VHS will only develop upon re-exposure to these innocent bystander antigens, possibly explaining why no persistent VHS is observed in our current study. Preliminary data seem to support this hypothesis[65, 66], but further experiments are clearly required.

In summary, our study shows that *C. rodentium* infection induces a transient VHS in C57BL/6 and Balb/c mice, that is more pronounced and prolonged in Th2-predominant Balb/c

mice, ~~indicating that a Th2 immune background may increase the susceptibility to develop PI-IBS~~. Although other strain-related differences, such as differences in nociception and behavior, may contribute, our data suggest that a Th2 background may represent an additional risk factor for prolonged PI-VHS. It should be emphasized though that PI-VHS was transient and thus other factors must be involved in the persistent VHS as observed in patients with PI-IBS. ~~An acute episode of stress in the post-infectious phase could not re-introduce VHS indicating that other mechanisms leading to persistent VHS, as observed in patients, must be involved.~~

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DISCLOSURES

The authors have no competing interests.

All authors read and approved the final version of the manuscript. MS: data acquisition, analysis and interpretation of data, writing and critical revision of the manuscript. TS, PE, FM

and AJ: data acquisition, analysis and interpretation of data. WM and BG: study supervision, obtaining funding and critical revision of the manuscript for important intellectual content. The study was published in abstract form at Digestive Disease Week 2014 (Chicago) as a poster (Tu1227).

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TABLES

Table 1 | Primer sequences for gene mRNA quantification by RT-qPCR.

Gene	Protein	Forward primer (5' -> 3')	Reverse primer (3' –> 5')
Actb	β-actin	CATTGCTGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
Il1b	IL-1β	GGGCCTCAAAGGAAAGAATC	TACCAGTTGGGGAACTCTGC
Il4	IL-4	AAGAACACCACAGAGAGTGAGCTC	TTTCAGTGATGTGGACTTGGACTC
Il6	IL-6	AAGTCGGAGGCTTAATTACACATGT	CCATTGCACAACTCTTTTCTCATTC
Il10	IL-10	AGAAGCATGGCCCTGAAATCAAGG	CTTGTAGACACCTTGGTCTTGGAG
Il13	IL-13	CACGGCCCCTTCTAATGAGG	CCTCTCCCCAGCAAAGTCTG
Il17	IL-17	ACCTCACACGAGGCACAAGT	AGCAGCAACAGCATCAGAGA
Ifng	IFN-γ	GCCATCAGCAACAACATAAGCGTC	CCACTCGGATGAGCTCATTGAATG
Tnf	TNF-α	CCCCAAAGGGATGAGAAGTT	CACTTGGTGGTTTGCTACGA
Ccl2	MCP-1	CCCCACTCACCTGCTGCTACT	GGCATCACAGTCCGAGTCACA
Kit	c-kit	TGGGAGCTCTTCTCCTTAGGAA	TGCTCCGGGCTGACCAT
Tpsb2	Tryptase β 2	GCAGCTAAGATGCTGAAGCG	CCTCATGTCCTCCCACGATG
Tpsab1	Tryptase α/β 1	TTGCTGACCCCAACAAGGTC	GGACGATGTAGAAGTCGGGG

FIGURE LEGENDS

Figure 1 | Effect of *Citrobacter rodentium* infection on body weight and inflammatory gene expression of infected C57BL/6 mice. A, Body weight change during 5 weeks following infection in C57BL/6 mice. Data are presented as mean ± SEM. 2-way ANOVA Bonferroni correction, * $p < 0.05$. **B**, Scatter plots of colonic inflammatory gene mRNA expression relative to β-actin in non-infected (non-inf, at 14d post-vehicle) and *C. rodentium* infected C57BL/6 mice at 10 days, 14 days PI and 5 weeks PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, PI = post-infection, TNF = tumor necrosis factor, w = week.

Figure 2 | Effect of *Citrobacter rodentium* infection on body weight and inflammatory gene expression of infected Balb/c mice. A, Body weight change during 5 weeks following infection in Balb/c mice. Data are presented as mean ± SEM. 2-way ANOVA Bonferroni correction, * $p < 0.05$, ** $p < 0.01$. **B**, Scatter plots of colonic inflammatory gene mRNA expression relative to β-actin in non-infected (non-inf, at 14d post-vehicle) and *C. rodentium* infected Balb/c mice at 10 days, 14 days PI and 5 weeks PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, PI = post-infection, TNF = tumor necrosis factor, w = week.

Figure 3 | Acute *C. rodentium* infection induces mild colonic inflammation in both C57BL/6 and Balb/c mice. H&E staining showing colonic sections at 10x enlargement in

non-infected and *C. rodentium* infected C57BL/6 (**A**) and Balb/c (**B**) mice with associated crypt length measurements at day 10 PI, day 14 PI and at 5 weeks PI. n = 4 – 7 mice/group. p unpaired t-test Welch's correction as indicated. The horizontal lines represent the mean ± SEM.

Figure 4 | Acute *C. rodentium* infection triggers transient VHS in both C57BL/6 and Balb/c mice that is not restored by acute water avoidance stress in the post-infectious phase. A-B Upper panel: VMR recordings in C57BL/6 (**A**) and Balb/c (**B**) mice measured before infection (pre-infection, black dotted line) and at 2 (blue full line) and 3 (orange full line) weeks PI. **A-B** Lower panel: VMR recordings measured at 4 weeks PI (grey full line) and at 5 (green full line) weeks PI following WAS. n = 4 – 7 mice/group, 2-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$. Data are presented as mean + SEM.

AUC of VMR responses measured throughout the whole experiment in *C. rodentium* infected C57BL/6 (**C**) and Balb/c (**D**) mice. The black horizontal lines represent the 95% percentile of the AUC of *C. rodentium* infected C57BL/6 and Balb/c mice measured prior to *C. rodentium* infection. Data are presented as mean ± SEM. p paired t-test, as indicated. AUC = area under the curve, hr = hour, PI = post-infection, pre-inf = pre-infection, VMR = visceromotor response, WAS = water avoidance stress, w = week.

SUPPLEMENTARY FIGURES (for online publication only)

Supplementary Fig. 1 | Acute *C. rodentium* infection induces subtle changes in inflammatory gene mRNA expression in the small intestine of C57BL/6 at 10 days post-infection. Scatter plots of small intestinal inflammatory gene mRNA expression relative to β-

actin in C57BL/6 (**A**) and Balb/c (**B**) mice at 10 days and 14 days PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, PI = post-infection, TNF = tumor necrosis factor.

Supplementary Fig. 2 | Comparison of colonic inflammatory gene mRNA expression between *C. rodentium* infected C57BL/6 and Balb/c mice. Scatter plots of colonic inflammatory mRNA expression in non-infected and *C. rodentium* infected C57BL/6 and Balb/c mice at different time points (as indicated). n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. BL/6 = C57BL/6, d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, w = week.

Supplementary Fig. 3 | Maximum VMR response at 2 weeks post-infection correlates with duration of hypersensitivity. Individual data showing the AUC of VMR responses from *C. rodentium* infected C57BL/6 and Balb/c mice (as indicated) measured up to 4 weeks post-infection. n = 5 - 7 mice/group.

Effect of genetic background and post-infectious stress on visceral sensitivity in *Citrobacter rodentium* infected mice

Running title: post-infectious visceral hypersensitivity

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ABSTRACT

Background: Infectious gastroenteritis is a major risk factor to develop post-infectious irritable bowel syndrome (PI-IBS). It remains unknown why only a subgroup of infected individuals develops PI-IBS. We hypothesize that immunogenetic predisposition is an important risk factor. Hence, we studied the effect of *Citrobacter rodentium*-infection on visceral sensitivity in Th1-predominant C57BL/6 and Th2-predominant Balb/c mice.

Methods: Eight weeks old mice were gavaged with *Citrobacter rodentium,* followed by 1 hour of water-avoidance stress (WAS) at 5 weeks PI. At 10, 14 days and 5 weeks PI, samples were assessed for histology and inflammatory gene expression by RT-qPCR. Visceral sensitivity was evaluated by visceromotor-response-recordings (VMR) to colorectal-distension.

Key results: *Citrobacter rodentium* evoked a comparable colonic inflammatory response at 14 days PI characterized by increased crypt length and upregulation of Th1/Th17 cytokine mRNA levels ($p_{uncorrected}<0.05$) in both C57BL/6 and Balb/c mice. At 5 weeks PI, inflammatory gene mRNA levels returned to baseline in both strains. The VMR was maximal at 14 days PI in C57BL/6 (150±47%;p=0.02) and Balb/c mice (243±52%;p=0.03). At 3 weeks PI, the VMR remained increased in Balb/c (176±23%; p=0.02), but returned to baseline in C57BL/6 mice. At 5 weeks PI, WAS could not re-introduce visceral hypersensitivity (VHS).

Conclusions&Inferences: *Citrobacter rodentium* infection induces transient VHS in C57BL/6 and Balb/c mice, which persisted one week longer in Balb/c mice. Although other strain-related differences may contribute, a Th2 background may represent a risk factor for prolonged PI-VHS. As PI-VHS is transient, other factors are crucial for persistent VHS development as observed in PI-IBS.

Key words: Irritable Bowel Syndrome, visceral hypersensitivity, citrobacter rodentium

KEY MESSAGES

General statement: Visceral hypersensitivity (VHS) is a hallmark of (post-infectious) irritable bowel syndrome (PI-IBS) but the underlying mechanisms remain largely unknown.

Aims/goals: We studied whether immunogenetic background and acute stress in the post-*Citrobacter rodentium* infectious phase influence the development of VHS.

Basic methodology: Visceral nociception was assessed by visceromotor-response-recordings (VMR) to colorectal distension. Colonic inflammation was evaluated by RT-qPCR and H&E staining.

Summary: In the acute infectious phase, *Citrobacter rodentium* evoked maximal visceral pain perception in Th1 and Th2 predominant C57BL/6 and Balb/c mice respectively. Visceral nociception remained increased in Balb/c but not in C57BL/6 mice at 3 weeks PI. Five weeks PI, inflammation was completely resolved and VMR returned to normal in both strains. Acute water avoidance stress could not re-introduce VHS, regardless of the immunogenetic background.

INTRODUCTION

Three to 31 % of individuals develop irritable bowel syndrome (IBS)[1] following an infectious gastroenteritis[2-11] and are referred to as post-infectious IBS patients (PI-IBS). Symptoms vary from patient to patient but typically include chronic abdominal pain, bloating and altered defecation patterns in the absence of an organic cause[12]. Visceral hypersensitivity, defined as increased sensitivity to visceral stimuli such as luminal distension, is one of the hallmarks of IBS[13] and can persist for years after the initial infection[14]. Up to date, it is unknown why only a subgroup of infected individuals will develop PI-IBS.

The risk to develop PI-IBS varies with the infectious agent[15-18] with *Campylobacter jejuni*, *Salmonella*, *Shigella* and *Escherichia coli* as main pathogens. Human *Escherichia coli* colitis can be modeled by the *Citrobacter rodentium* murine model of self-limiting colitis, as *C. rodentium* shares 67% of its genes with the human enteropathogenic and enterohaemorrhagic *E. coli*[19, 20]. Based on these observations, *C. rodentium* infection may represent a potential model of PI-IBS. Previously, Ibeakanma C. et al. showed that *C. rodentium* infection in the Th1 predominant mouse strain C57BL/6 mice, evoked hyperexcitability of colonic dorsal root ganglia (DRG) neurons and increased afferent nerve firing that persisted until 30 days PI[21]. Stress concurrently with the infection enhanced neuronal excitability, while repeated water avoidance stress in the PI phase produced no greater enhancement than stress applied alone[21, 22], indicating that stress at the time of infection seems to increase the risk to develop post-infectious visceral hypersensitivity.

Microscopic inflammation has been well documented in PI-IBS and is believed to underlie PI-IBS pathophysiology[2, 23-25]. Serial rectal biopsies taken from patients who developed IBS after Campylobacter *jejuni* gastroenteritis showed a persistent inflammatory infiltrate, with an increase in enterochromaffin cells and T lymphocyte cell counts[2]. Increased mast cell

numbers have been observed in PI-IBS[24] and non PI-IBS[26], showing significant correlations between IBS severity and mast cell counts, spontaneous mast cell tryptase release and colonic permeability[26]. Additional evidence includes upregulation of the pro-inflammatory cytokine interleukin-1β (IL-1β) in rectal biopsies of PI-IBS patients[27], and increased pro-inflammatory cytokine release, f.e. TNF-α, IL-1β and IL-6, by peripheral blood mononuclear cells[28]. Based on the knowledge that mast cells play a key role in IBS and are classically involved in a Th2 immune response, we speculate that the immunogenetic background of the host may be an important component dictating the nature of the immune response and the subsequent development of PI-IBS.

To gain insight into the role of immunogenetic background as a risk to develop VHS after an infection, we assessed the course of infection and visceral sensitivity in *C. rodentium* infected Th1 predominant C57BL/6 and Th2 predominant Balb/c mice. Moreover, we assessed if stress in the PI phase can re-initiate visceral hypersensitivity.

MATERIALS AND METHODS

All experiments were conducted in accordance with the institutional guidelines and approved by the animal ethical committee of the KU Leuven (protocol numbers P179/2009 and P109/2010).

Animals

Six weeks old male C57BL/6 and Balb/c mice were purchased from Janvier (Saint-Berthevin Cedex, France) and left undisturbed for at least 1 week for acclimatization. Animals were maintained under a 14:10h dark/light cycle, at a temperature of 20-22 °C, provided with food and water *ad libitum*.

Citrobacter rodentium (C. rodentium) infection

C. rodentium (DBS100, ATCC® 51459™, Teddington, United Kingdom) was cultured overnight in Luria-Bertani broth (LB) medium (MP Biomedicals, Drogenbos, Belgium) (37 °C, 200 rpm). Fifteen hours later, the bacteria were centrifuged (4 °C, 2000 rpm, 10 minutes) and fresh LB medium was added to dissolve the pellet. Eight weeks old mice were infected by oral gavage of 3 x 10^{10} colony forming units *C. rodentium* or sterile 0.9 % NaCl (B. Braun Medical NV/SA, Diegem, Belgium) followed by intraperitoneal (i.p.) injection of 200 µl sterile 0.9 % NaCl to prevent dehydration[29, 30].

Water avoidance stress (WAS) model

At 5 weeks post-infection, a bucket with a platform of 40 mm diameter was filled with fresh room temperature water (20 °C) up to 1 cm of the top of the platform. Mice were placed on the platform for 1 hour and the visceromotor response was measured 24 hours later[31].

Telemetric Visceromotor Response (VMR) recordings

Mice of at least 20 grams were implanted with Physiotel ETA-F10 telemetric transmitters (Data Sciences International, MC s'Hertogenbosch, The Netherlands). Hereto, mice were anaesthetized by i.p. injection of 20 mg/kg ketamine (Nimatek, Eurovet Animal Health B.V., AE Bladel, The Netherlands) and 1 mg/kg xylazine (Rompun 2 %, Bayer, Diegem, Belgium) and placed on a heating pad (± 30 °C). The telemetric transmitter was inserted in the abdominal cavity, the electrodes were tunneled through the abdominal wall using a 18G needle (Terumo Europe n.v., Leuven, Belgium) and the non-insulated tips were sutured in parallel (± 5 mm apart) into the left external abdominal oblique muscle. After surgery, mice recovered for 12 hours on a heating pad where after they were left undisturbed for 10

consecutive days[32]. The radio telemetry experimental setup for measurement of the visceromotor response (VMR) to colorectal distension (CRD) in mice was adapted from[33, 34].

Colorectal distensions were performed to evoke abdominal cramping as read-out of visceral nociception. Hereto, a distension catheter (Fogarty catheter for arterial embolectomy, 4F; Edwards Lifesciences, Breda, The Netherlands) was inserted into the colon (3 cm from the rectum) of conscious, non-restraint, mice and distended with volumes progressively increasing from 20 µL to 80 µL, with each step lasting 10 sec and separated by 5 min non-distension periods in-between[35]. The VMR responses were measured and quantified using Acknowledge 3.2.6 software (BIOPAC Systems Inc., Goleta, California, USA). For analysis, the mean value of the resting EMG signal 10 sec prior to distension (i.e. basal activity) was subtracted from the mean value of the electromyography signal evoked during the 10 sec distension. Data are presented as % VMR response ± SEM relative to maximum nociception response before infection (i.e. 80 µl distension is set at 100 %) or as area under the curve of the VMR responses.

Evaluation of colonic inflammation by real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 30-50 mg intestinal tissue using the RNeasy Minikit (Qiagen GmBH, Hilden, Germany) according to the manufacturer's instructions. cDNA of 2 µg total RNA was transcribed by the qScript cDNA supermix (Quanta Biosciences, Gaithersburg, Maryland, USA) according to manufacturers' instructions.

The primer sequences used to quantify inflammatory gene mRNA are listed in Table 1. Ten µl reaction mix per well was loaded onto a LightCycler® 480 multiwell plate 96 (Roche GmBH, Mannheim, Germany) containing 2.5 µl of each cDNA sample together with 5 µl FastStart

Essential DNA Green Master (Roche GmBH, Mannheim, Germany), 0.2 µl oligonucleotides (10 µM) and 2.3 µl RNase Free Water (Applied Biosystems, Halle, Belgium). Gene expression was normalized to an endogenous reference gene, β-actin. Relative gene expression was calculated as $2^{-\Delta\Delta Ct}$ [36] and data are presented as relative expression ± standard error of the mean (SEM).

Histopathology assessment

At 10, 14 days and 5 weeks post-infection, full thickness intestinal tissue samples were freshly frozen in Tissue-Tek O.C.T.™ compound (Sakura Finetek Europe B.V., AV Alphen aan den Rijn, The Netherlands). Eight µm cryosections were cut and stained with heamatoxylin & eosin (H&E). Slides were reviewed using an Olympus BX41 light microscope (Aartselaar, Belgium) and scored blinded. H&E staining was performed to assess the degree of colitis using crypt length, crypt space and muscle thickness to address epithelial changes and overall mucosal architecture of each specimen[37]. All measurements were performed using Cell^F software (Aartselaar, Belgium). Mean crypt height was calculated from 5-10 individual measurements of the lengths of all well oriented crypts on each specimen slide.

Statistical analysis

All statistical analyses were performed with Graphpad Prism software (GraphPad Software, San Diego, California, USA). Non-infected versus *C. rodentium* infected groups were compared by Unpaired t-test with Welch's correction. Results are presented after Bonferroni correction for multiple testing (for RT-qPCR data based on 13 genes, $p_{uncorrected}<0.004$ was considered to be significant). VMR recordings were analyzed by 2-way ANOVA with

Bonferroni post-hoc correction. A p value < 0.05 was considered significant. Data are presented as mean + SEM.

RESULTS

C. rodentium infection evokes transient colonic inflammation in C57BL/6 and Balb/c mice

All animals were monitored daily and the majority of animals (85–90%) exhibited signs of illness (e.g. decreased activity) for 2–3 days following infection but recovered quickly. None of the animals died. Infected C57BL/6 mice lost 5% of their initial body weight compared to 2% in Balb/c mice within the first 4 days post-infection (PI). Up to 2 weeks PI, infected C57BL/6 (Fig. 1A) and Balb/c (Fig. 2A) did not reach the body weight of the non-infected controls.

To identify potential differences in the degree of inflammation evoked by *C. rodentium*, inflammatory marker mRNA expression and histology were assessed in colon and small intestine at 10 and 14 days PI. Small intestinal *IFN-γ*, *TNF-α* and *IL-1β* mRNA expression was increased in *C. rodentium* infected C57BL/6 mice at 10 days PI but this increase was not significant after correction for multiple testing (Supplementary Fig. 1A). In contrast, *MCP-1* mRNA levels were significantly decreased (p unpaired t-test Welch's correction = 0.0001) at 10 days PI in the small intestine of infected Balb/c mice (Supplementary Fig. 1B).

At 10 days PI, colonic *c-kit* mRNA expression was significantly decreased in infected C57BL/6 mice compared to non-infected controls (p unpaired t-test Welch's correction = 0.0037; Fig. 1B). *MCP-1*–and *IL-10* mRNA expression was increased in infected C57BL/6 mice, but did not remain significant after correction for multiple testing (Fig. 1B). At 14 days PI, there was a tendency towards increased *IL-1β, IL-17* and *IL-10* mRNA expression while *c-*

kit mRNA expression was decreased in infected C57BL/6 mice, these results did not remain significant after correction for multiple testing (Fig. 1B).

In contrast, infected Balb/c mice showed decreased colonic mRNA levels of *MCP-1* and increased *IL-10* mRNA expression compared to non-infected controls at 10 days PI (p unpaired t-test Welch's correction < 0.004; Fig. 2B). *IFN-γ, TNF-α*, *IL-1β* and *IL-17* mRNA were also upregulated, but could not withstand correction for multiple testing. At 14 days PI, *TNF-α, IL-17* and *IL-10* mRNA levels were increased (p unpaired t-test Welch's correction <0.004, Fig. 2B). Of note, colonic mast cell *tryptase a/b* mRNA expression was increased at 10 and 14 days PI in infected Balb/c mice, but could not withstand correction for multiple testing (Fig. 2B).

Comparison of colonic inflammatory gene expression between infected C57BL/6 and Balb/c mice in the acute inflammatory phase revealed only increased *tryptase a/b* levels in Balb/c mice (unpaired t-test Welch's correction p = 0.004) (Supplementary Fig. 2) and a tendency for increased *IL-17* mRNA and decreased *IL-6* in Balb/c mice, but this could not withstand correction for multiple testing. At 5 weeks PI, *IFN-γ* and *IL-10* mRNA levels were significantly lower in infected Balb/c compared to infected C57BL/6 mice (Supplementary Fig. 2).

H&E staining was performed to assess the degree of colitis using crypt length, crypt space and muscle thickness to address epithelial changes and overall mucosal architecture. Histology of the colon at 10 days PI shows signs of colitis as reflected by increased crypt length in both infected C57BL/6 (Fig. 3A) and Balb/c (Fig. 3B) mice compared to non-infected controls (p unpaired t-test Welch's correction = 0.029 and p <0.0001, respectively), an effect that was more pronounced at PI day 14 (Fig. 3A, B). No changes in muscle thickness or space between the crypts were observed between non-infected and infected mice,

irrespective of genetic immune background (data not shown). Based on the inflammatory cell infiltrate in mucosa and submucosa, mild hyperplasia and minimal goblet cell loss, we can conclude that *C. rodentium* induces a mild colonic inflammation in both mouse strains.

At 5 weeks PI, in the absence of histological changes (Fig. 3A, B), infected C57BL/6 but not Balb/c mice still showed a tendency, albeit not statistically significant after multiple testing correction, towards increased colonic inflammatory mRNA expression of *IL-1β, IL-6, IFN-γ* and *IL-17* compared to non-infected controls (Fig. 2B).

Taken together, *C. rodentium* evokes a transient mild inflammatory response characterized by upregulation of inflammatory cytokine mRNA in both mouse strains at 10 and 14 days PI, with pronounced *IL-17* mRNA upregulation.

C. rodentium infection induces transient VHS in C57BL/6 and Balb/c mice

Colorectal sensitivity was assessed by VMR at 2, 3 and 4 weeks PI and 24 hours post-WAS at 5 weeks PI. At 2 weeks PI, in the presence of colonic inflammation, visceral sensitivity was significantly increased compared to baseline in both infected C57BL/6 (Fig. 4A) and Balb/c (Fig. 4B) mice. The response to colorectal distension (increase in visceral nociception at the maximum distension volume compared to pre-infection) was increased by 50 + 16% in C57BL/6 (p 2-wayANOVA=0.02) and by 143 + 31% in Balb/c (p 2-wayANOVA=0.03) mice. At 3 weeks PI, visceral sensitivity to colorectal distension returned to baseline levels in C57BL/6 mice, while the VMR response to colorectal distension was still significantly increased by 76 + 14% in Balb/c mice (p 2-wayANOVA=0.02). Only Balb/c mice with the highest VMR response at 2 weeks PI remained, to a lower extent, hypersensitive at 3 weeks PI (Supplementary Fig. 3). Visceral sensitivity normalized at 4 weeks PI in both mouse strains (Fig 4 A-D).

As stress may exacerbate or re-initiate visceral sensitivity[22, 38, 39], we next assessed the effect of acute water avoidance stress (WAS) on post-infectious visceral sensitivity. One hour of WAS did not re-install VHS at 5 weeks PI (Fig. 4A-D). Visceral sensitivity to colorectal distension was similar to that before the infection, for both mouse strains (Fig. 4A-D).

DISCUSSION

In humans, it remains unclear why only a subgroup of infected individuals develops long-term PI-IBS. Based on the importance of mast cells in IBS symptom generation[25, 40-42], we hypothesized that a Th2 immune background may increase the risk to develop PI-IBS and used *Citrobacter rodentium* as a murine model to study our hypothesis. In the present study, we evaluated the role of *C. rodentium*-induced inflammation and acute stress on visceral sensory function in Th1-predominant C57BL/6 and Th2-predominant Balb/c mice. *C. rodentium* induced a self-limiting colitis in both strains with induction of colonic inflammation and increased visceral sensitivity to colorectal distension at 2 weeks post-infection. The increase in visceral nociception was transient and lasted 1 week longer in the Th2-predominant Balb/c mice. An episode of acute water avoidance stress (WAS) did not re-initiate PI-VHS irrespective of the immunogenetic background. These results suggest that a Th2-predominant immunogenetic background may represent one of the risk factors to develop prolonged abnormal visceral nociception following an episode of infectious gastroenteritis. Of note, other strain-related factors, such as differences in nociception and behavior, may undoubtedly contribute as well.

In mice, *C. rodentium* is known to induce an acute, self-limiting colitis, histologically associated with crypt hyperplasia and goblet cell depletion[43, 44]. The infection serves as a model for human infectious gastroenteritis induced by enteropathogenic *Escherichia coli*, a well-known trigger for PI-IBS in humans[45]. Upon infection, *C. rodentium* transiently

colonizes the distal colon with a peak of infection around 10 – 14 days PI in both C57BL/6[21] and Balb/c mice[46]. In line, we showed that *C. rodentium* induced a transient colonic inflammation, characterized by increased cytokine mRNA levels, irrespective of the genetic background, with overt inflammation at 14 days post-infection. The more pronounced colonic expression of *IFN-γ*/*IL-17*, identified as crucial players for host defense against infection[47] at day 14 PI indicates that the peak of infection for both Th1 and Th2 predominant mice lays around 14 days post-infection. Inflammatory cytokine mRNA expression was however not significantly different between the two mouse strains, except for increased *tryptase a/b* mRNA levels in infected Balb/c mice compared to infected C57BL/6 mice. The peak of infection/inflammation was associated with an increase in visceral nociception to colorectal distension in both strains at 2 weeks PI. The duration of increased nociception differed however, i.e. Balb/c mice showed increased VMR responses to colorectal distension up to 3 weeks PI (76% increase) while visceral perception of C57BL/6 mice was already normalized.

Of interest, we noticed that mainly mice with a very high VMR response at 2 weeks PI (in particular Balb/c mice) remained VHS at 3 weeks PI (Supplementary Fig. 3), indicating that the magnitude of the VMR response at 2 weeks PI may be associated with a slower recovery and increased duration of the aberrant nociceptive response. One potential explanation may be the difference in immunogenetic background leading to more pronounced mast cell activation in the Th2 prone Balb/c mice, as suggested by increased *tryptase a/b* mRNA expression in the acute infectious phase in Balb/c mice compared to infected C57BL/6 mice. However, the degree of upregulation was relatively small questioning its physiological relevance. Moreover, Th2-associated cytokine expression did not differ between *C. rodentium* infected C57BL/6 and Balb/c mice, suggesting that other factors contribute to the prolonged VHS observed in infected Balb/c mice. In fact, there are indeed documented strain differences with

respect to behavioral[48, 49] and nociceptive tests[50, 51], most likely contributing to the prolonged VHS in Balb/c mice. It should also be emphasized though that the VHS observed in both strains completely normalized during the PI phase (i.e. at 4 weeks PI), while patients with PI-IBS continue to have symptoms for several years following the infectious episode. These findings are in accordance with other studies, showing no increased VMR response in C57BL/6 mice 30 days PI[22]. Hence, other mechanisms may be critical for the development of chronic VHS.

Psychological comorbidities such as stress, anxiety, depression and adverse early-life events are known to induce and/or exacerbate IBS symptoms[52-57]. Previously, van den Wijngaard et al.[58] showed long-lasting VHS in response to an episode of acute stress, induced by water-avoidance stress (WAS), in a rat model of maternal separation[58, 59]. Therefore, we evaluated if a previous gastrointestinal infection would increase the risk to develop VHS in response to WAS. In the present study however, acute WAS at 5 weeks PI did not recreate VHS, irrespective of genetic background. Our data therefore seem to indicate that stress applied after an intestinal infection is not a major trigger to develop long-lasting VHS. Indeed, Spreadbury et al.[60] showed that chronic psychological stress after clearance of the infection did not have an additional effect on neuronal excitability compared to non-infected mice exposed to the same stressor[60]. Nevertheless, these data confirm that the bacterial infection *per se* does not alter visceral perception. It should be stressed though that psychological co-morbidity *prior* to and not after clearance of the infection is associated with an increased risk to develop IBS[22, 60, 61]. In line, stress during or before *C. rodentium* infection results in an exaggerated peripheral nociceptive signaling compared to *C. rodentium* alone[22] and to enhanced excitability of dorsal root ganglion neurons compared to non-infected controls[60]. Altogether, these data highlight the importance of the timing of stress relative to the infection.

This was indeed further corroborated by our recent study showing that psychological comorbidity prior to or during a gastrointestinal infection predisposes individuals to develop IBS. Of note, we showed that the type of immune response raised against the infection is associated with the risk to develop IBS. Patients who developed a Th2-predominant cytokine profile at the time of infection had an increased risk of PI-IBS 1 year later[62]. These data together with our current findings seem to support the hypothesis that the immunogenetic background may, at least to some extent, contribute to the risk to develop PI-IBS. Nevertheless, the duration of VHS in our murine PI model is rather short lasting, so clearly other factors must be involved.

Of interest, recent evidence suggests that food allergens may be involved in IBS. Not only do more than 60% of patients with IBS report the onset or worsening of symptoms after meals[63], submucosal instillation of food antigens in the duodenum was recently shown to evoke a local reaction with an instant influx of inflammatory cells and increased secretion[64]. Although the exact mechanism underlying these phenomena remains to be determined, one may speculate that an aberrant immune response to food antigens could be involved. Currently, we are investigating the hypothesis that prolonged VHS following a gastrointestinal infection may result from recurrent mast cell activation due to an aberrant immune response mounted against harmless intraluminal antigens present at the time of infection. If true, VHS will only develop upon re-exposure to these innocent bystander antigens, possibly explaining why no persistent VHS is observed in our current study. Preliminary data seem to support this hypothesis[65, 66], but further experiments are clearly required.

In summary, our study shows that *C. rodentium* infection induces a transient VHS in C57BL/6 and Balb/c mice, that is more pronounced and prolonged in Th2-predominant Balb/c mice. Although other strain-related differences, such as differences in nociception and behavior, may contribute, our data suggest that a Th2 background may represent an additional

risk factor for prolonged PI-VHS. It should be emphasized though that PI-VHS was transient and thus other factors must be involved in the persistent VHS as observed in patients with PI-IBS.

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DISCLOSURES

The authors have no competing interests.

All authors read and approved the final version of the manuscript. MS: data acquisition, analysis and interpretation of data, writing and critical revision of the manuscript. TS, PE, FM and AJ: data acquisition, analysis and interpretation of data. WM and BG: study supervision, obtaining funding and critical revision of the manuscript for important intellectual content. The study was published in abstract form at Digestive Disease Week 2014 (Chicago) as a poster (Tu1227).

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TABLES

Table 1 | Primer sequences for gene mRNA quantification by RT-qPCR.

Gene	Protein	Forward primer (5' -> 3')	Reverse primer (3' –> 5')
Actb	β-actin	CATTGCTGACAGGATGCAGAA	GCTGATCCACATCTGCTGGAA
Il1b	IL-1β	GGGCCTCAAAGGAAAGAATC	TACCAGTTGGGGAACTCTGC
Il4	IL-4	AAGAACACCACAGAGAGTGAGCTC	TTTCAGTGATGTGGACTTGGACTC
Il6	IL-6	AAGTCGGAGGCTTAATTACACATGT	CCATTGCACAACTCTTTTCTCATTC
Il10	IL-10	AGAAGCATGGCCCTGAAATCAAGG	CTTGTAGACACCTTGGTCTTGGAG
Il13	IL-13	CACGGCCCCTTCTAATGAGG	CCTCTCCCCAGCAAAGTCTG
Il17	IL-17	ACCTCACACGAGGCACAAGT	AGCAGCAACAGCATCAGAGA
Ifng	IFN-γ	GCCATCAGCAACAACATAAGCGTC	CCACTCGGATGAGCTCATTGAATG
Tnf	TNF-α	CCCCAAAGGGATGAGAAGTT	CACTTGGTGGTTTGCTACGA
Ccl2	MCP-1	CCCCACTCACCTGCTGCTACT	GGCATCACAGTCCGAGTCACA
Kit	c-kit	TGGGAGCTCTTCTCCTTAGGAA	TGCTCCGGGCTGACCAT
Tpsb2	Tryptase β 2	GCAGCTAAGATGCTGAAGCG	CCTCATGTCCTCCCACGATG
Tpsab1	Tryptase α/β 1	TTGCTGACCCCAACAAGGTC	GGACGATGTAGAAGTCGGGG

FIGURE LEGENDS

Figure 1 | Effect of *Citrobacter rodentium* infection on body weight and inflammatory gene expression of infected C57BL/6 mice. A, Body weight change during 5 weeks following infection in C57BL/6 mice. Data are presented as mean ± SEM. 2-way ANOVA Bonferroni correction, * $p < 0.05$. **B**, Scatter plots of colonic inflammatory gene mRNA expression relative to β-actin in non-infected (non-inf, at 14d post-vehicle) and *C. rodentium* infected C57BL/6 mice at 10 days, 14 days PI and 5 weeks PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, PI = post-infection, TNF = tumor necrosis factor, w = week.

Figure 2 | Effect of *Citrobacter rodentium* infection on body weight and inflammatory gene expression of infected Balb/c mice. A, Body weight change during 5 weeks following infection in Balb/c mice. Data are presented as mean ± SEM. 2-way ANOVA Bonferroni correction, * $p < 0.05$, ** $p < 0.01$. **B**, Scatter plots of colonic inflammatory gene mRNA expression relative to β-actin in non-infected (non-inf, at 14d post-vehicle) and *C. rodentium* infected Balb/c mice at 10 days, 14 days PI and 5 weeks PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, PI = post-infection, TNF = tumor necrosis factor, w = week.

Figure 3 | Acute *C. rodentium* infection induces mild colonic inflammation in both C57BL/6 and Balb/c mice. H&E staining showing colonic sections at 10x enlargement in

non-infected and *C. rodentium* infected C57BL/6 (**A**) and Balb/c (**B**) mice with associated crypt length measurements at day 10 PI, day 14 PI and at 5 weeks PI. n = 4 – 7 mice/group. p unpaired t-test Welch's correction as indicated. The horizontal lines represent the mean ± SEM.

Figure 4 | Acute *C. rodentium* infection triggers transient VHS in both C57BL/6 and Balb/c mice that is not restored by acute water avoidance stress in the post-infectious phase. A-B Upper panel: VMR recordings in C57BL/6 (**A**) and Balb/c (**B**) mice measured before infection (pre-infection, black dotted line) and at 2 (blue full line) and 3 (orange full line) weeks PI. **A-B** Lower panel: VMR recordings measured at 4 weeks PI (grey full line) and at 5 (green full line) weeks PI following WAS. n = 4 – 7 mice/group, 2-way ANOVA with Bonferroni correction, * $p < 0.05$, ** $p < 0.01$. Data are presented as mean + SEM.

AUC of VMR responses measured throughout the whole experiment in *C. rodentium* infected C57BL/6 (**C**) and Balb/c (**D**) mice. The black horizontal lines represent the 95% percentile of the AUC of *C. rodentium* infected C57BL/6 and Balb/c mice measured prior to *C. rodentium* infection. Data are presented as mean ± SEM. p paired t-test, as indicated. AUC = area under the curve, hr = hour, PI = post-infection, pre-inf = pre-infection, VMR = visceromotor response, WAS = water avoidance stress, w = week.

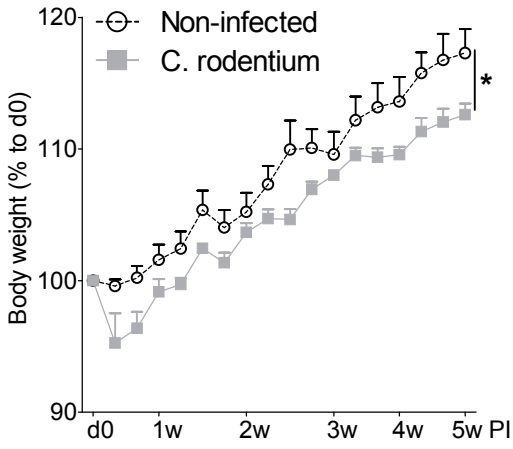
SUPPLEMENTARY FIGURES (for online publication only)

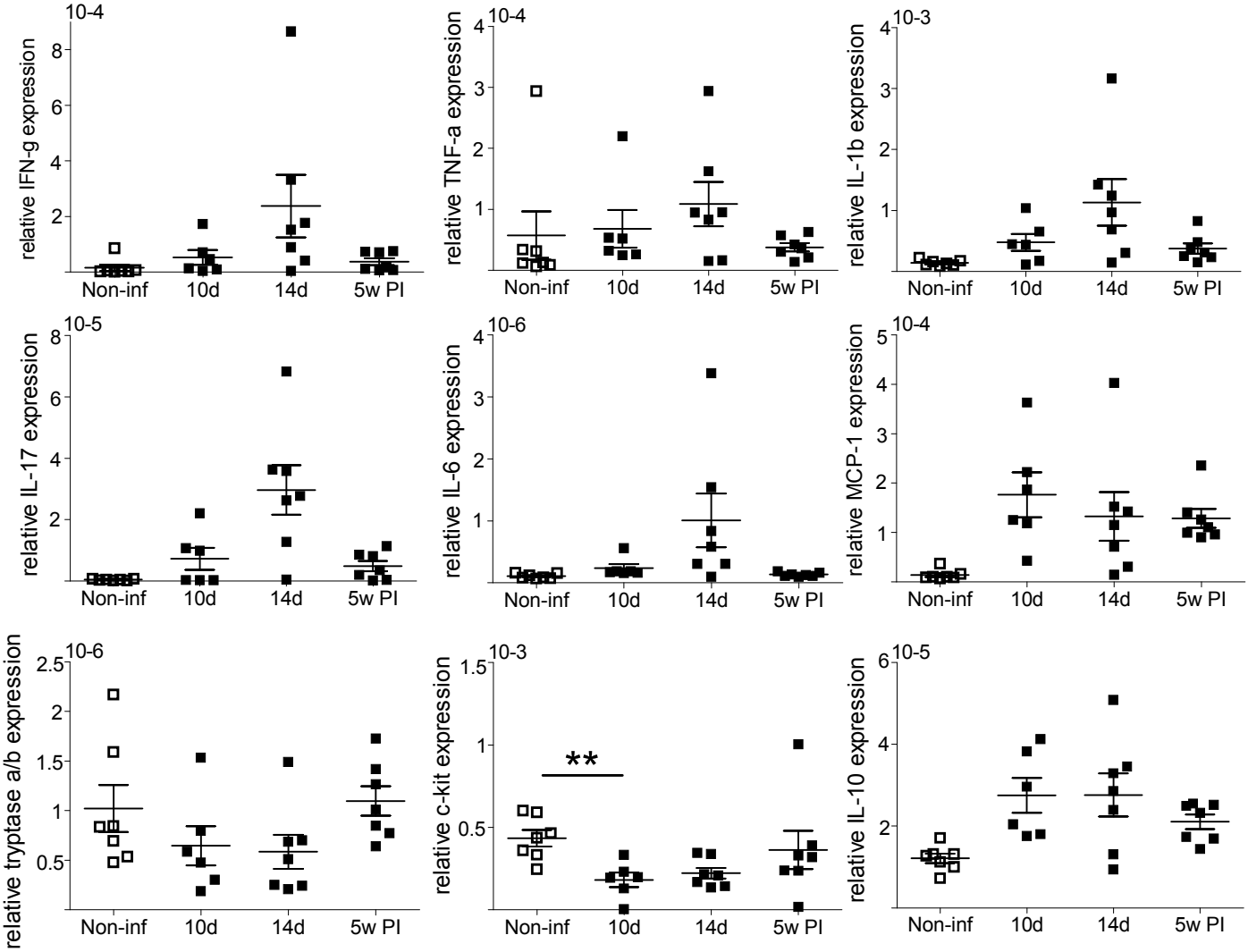
Supplementary Fig. 1 | Acute *C. rodentium* infection induces subtle changes in inflammatory gene mRNA expression in the small intestine of C57BL/6 at 10 days post-infection. Scatter plots of small intestinal inflammatory gene mRNA expression relative to β-

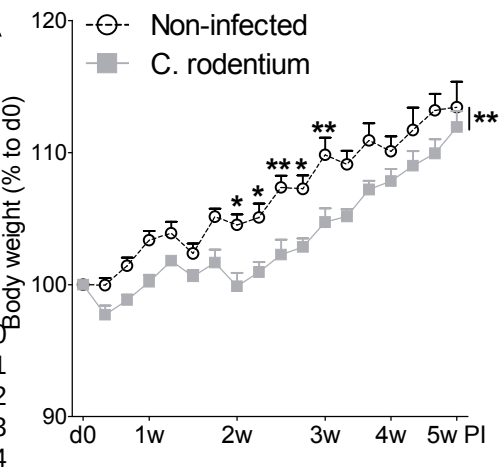
actin in C57BL/6 (**A**) and Balb/c (**B**) mice at 10 days and 14 days PI. n = 6 – 7 mice/group, unpaired t-test Welch's correction, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, PI = post-infection, TNF = tumor necrosis factor.

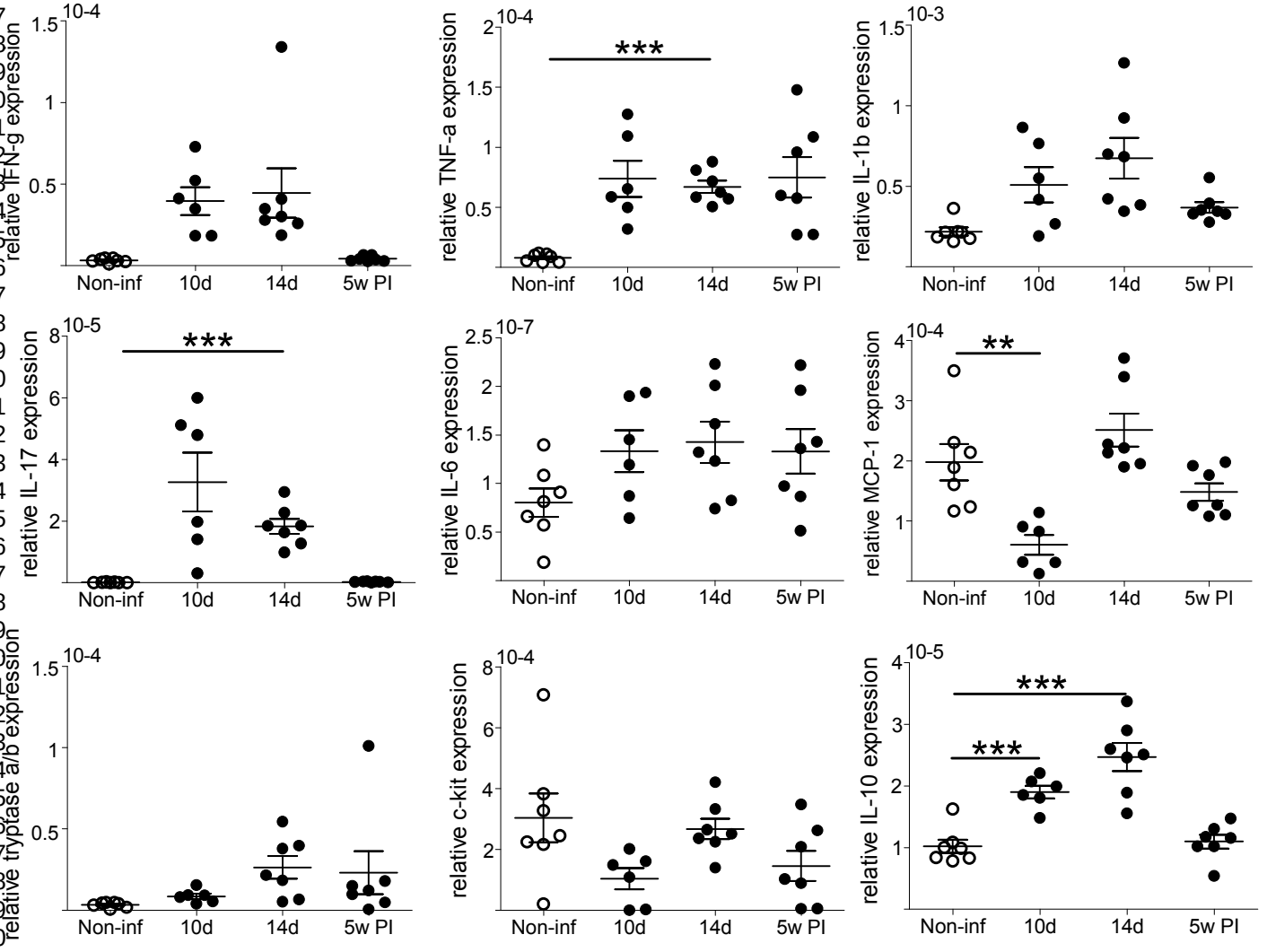
Supplementary Fig. 2 | Comparison of colonic inflammatory gene mRNA expression between *C. rodentium* infected C57BL/6 and Balb/c mice. Scatter plots of colonic inflammatory mRNA expression in non-infected and *C. rodentium* infected C57BL/6 and Balb/c mice at different time points (as indicated). n = 6 – 7 mice/group, unpaired t-test Welch's correction, ** $p < 0.004$, *** $p < 0.001$. The horizontal lines represent the mean ± SEM. BL/6 = C57BL/6, d = day, IL = interleukin, IFN = interferon, MCP1 = monocyte chemotactic protein 1, non-inf = non-infected, w = week.

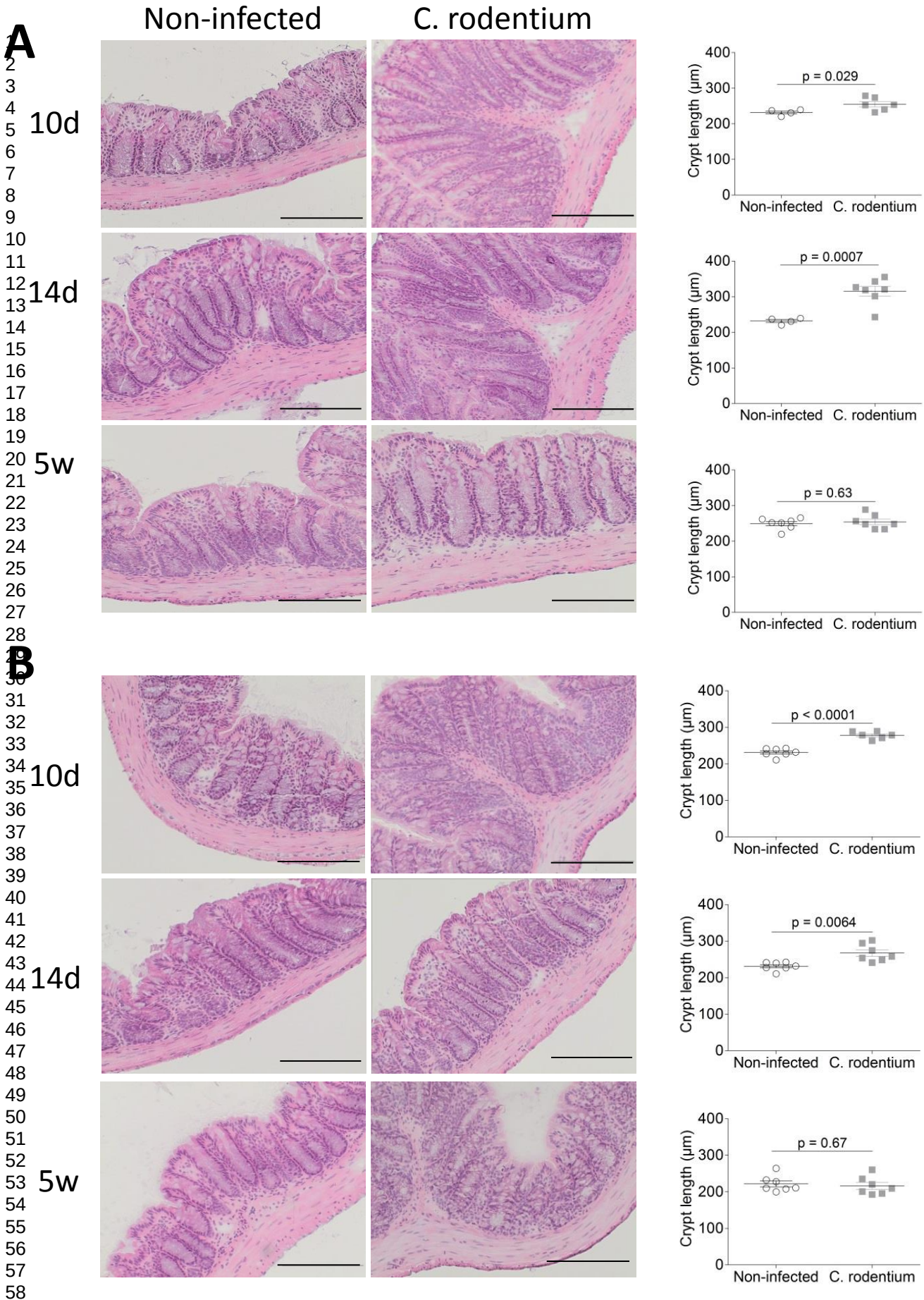
Supplementary Fig. 3 | Maximum VMR response at 2 weeks post-infection correlates with duration of hypersensitivity. Individual data showing the AUC of VMR responses from *C. rodentium* infected C57BL/6 and Balb/c mice (as indicated) measured up to 4 weeks post-infection. n = 5 - 7 mice/group.

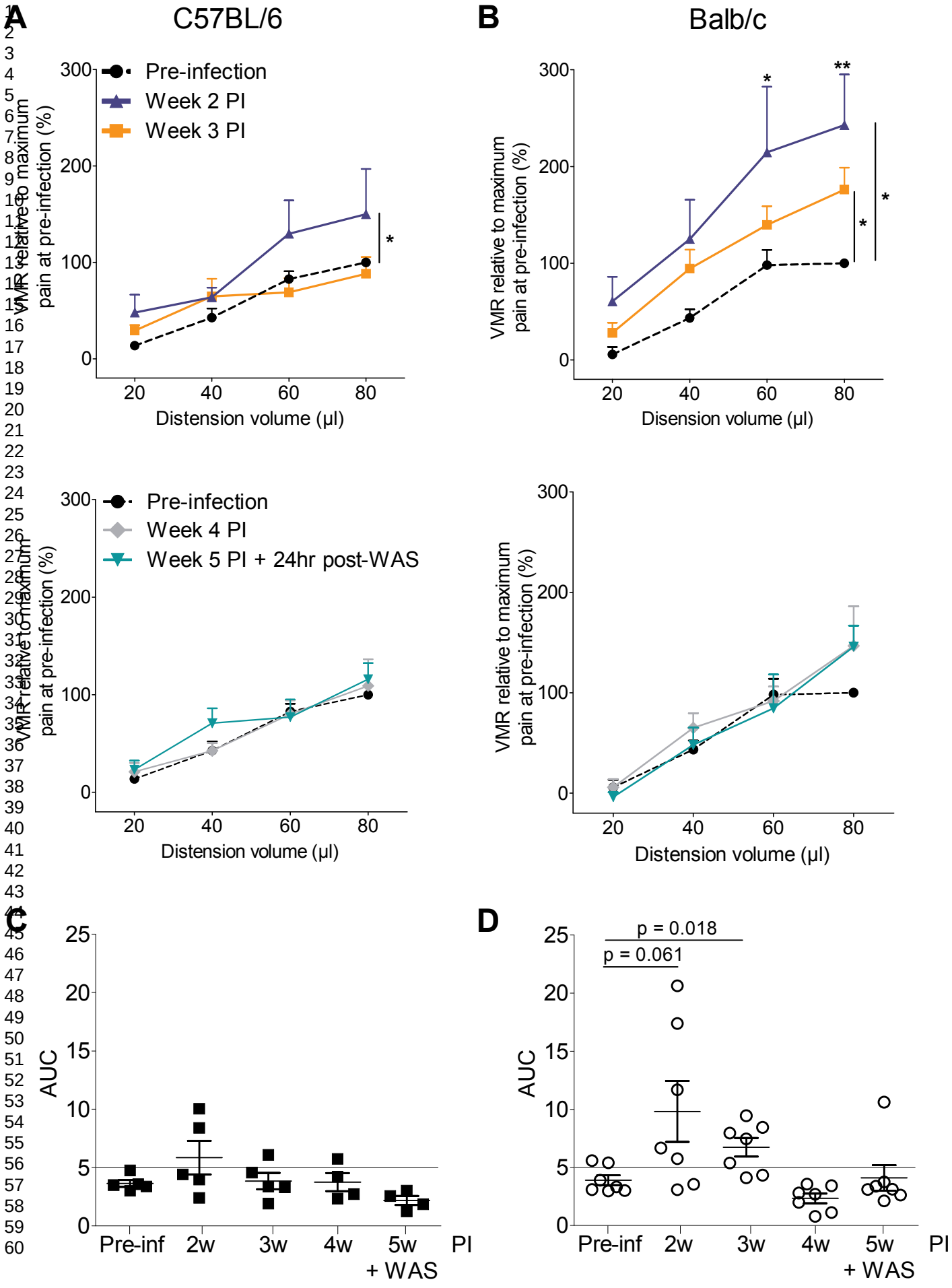

A
Non-infected
C. rodentium
Body weight (% to d0)
120
110
100
90
*
d0
1w
2w
3w
4w
5w PI

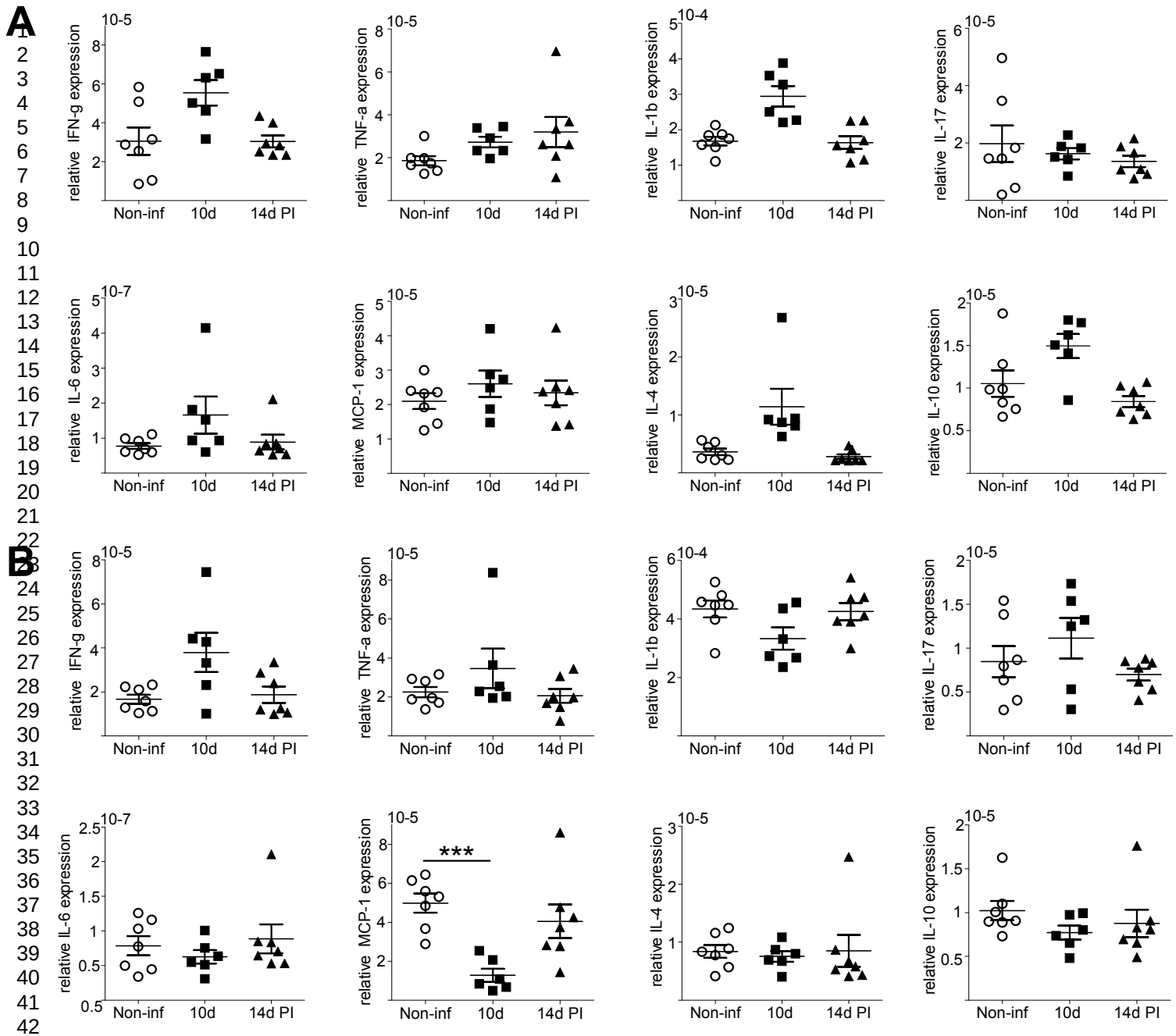

B
relative IFN-g expression
10-4
relative TNF-a expression
10-4
relative IL-1b expression
10-3
relative IL-17 expression
10-5
relative IL-6 expression
10-6
relative MCP-1 expression
10-4
relative tryptase a/b expression
10-6
relative c-kit expression
10-3
**
relative IL-10 expression
10-5
Non-inf
10d
14d
5w PI


A
Non-infected
C. rodentium
Body weight (% to d0)
120
110
100
90
d0
1w
2w
3w
4w
5w PI
*
*
***
**
**

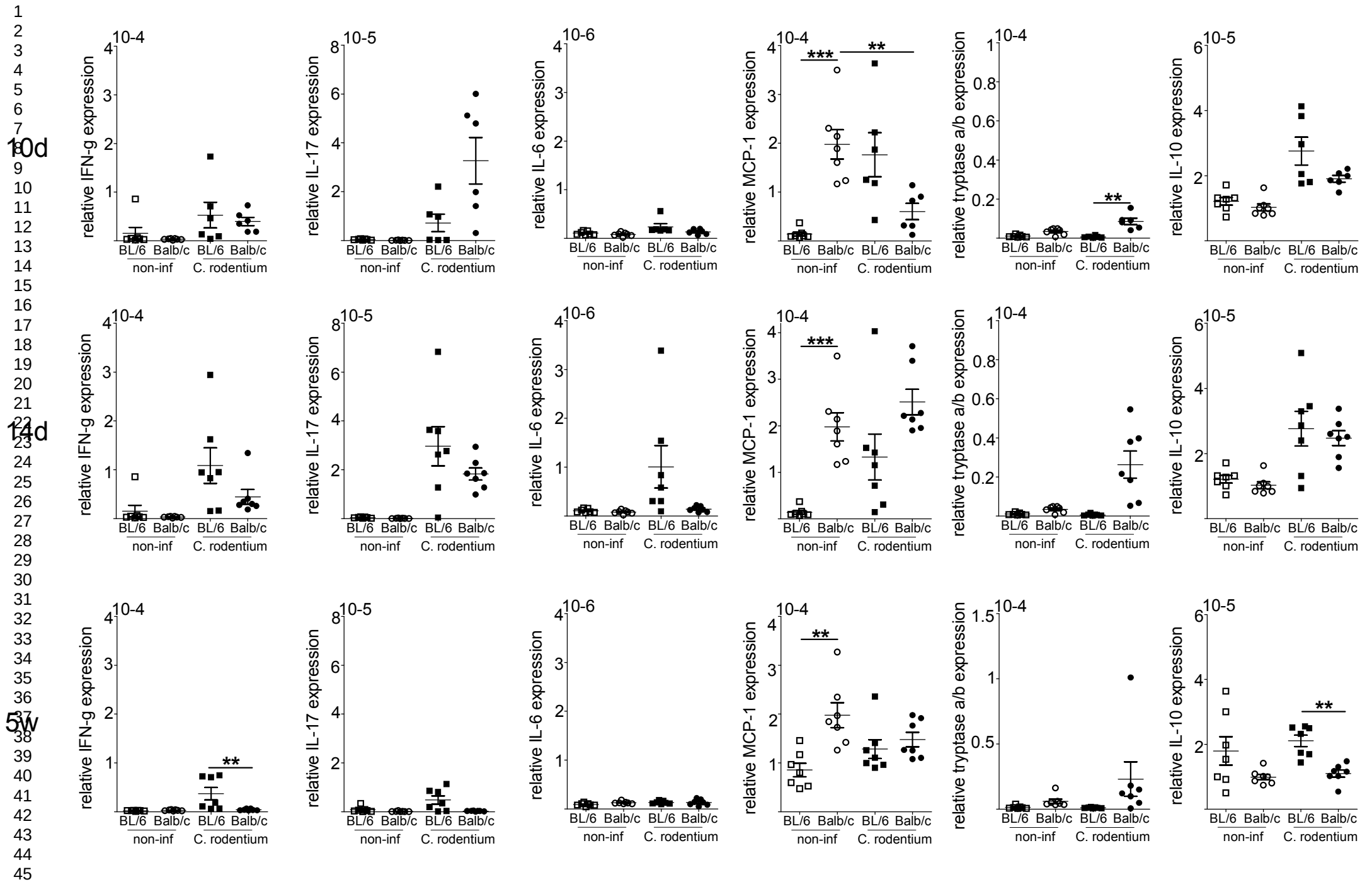

B
relative IFN-g expression
10-4
relative TNF-a expression
10-4
***
relative IL-1b expression
10-3
relative IL-17 expression
10-5
***
relative IL-6 expression
10-7
relative MCP-1 expression
10-4
**
relative tryptase a/b expression
10-4
relative c-kit expression
10-4
relative IL-10 expression
10-5
***
***
Non-inf
10d
14d
5w PI

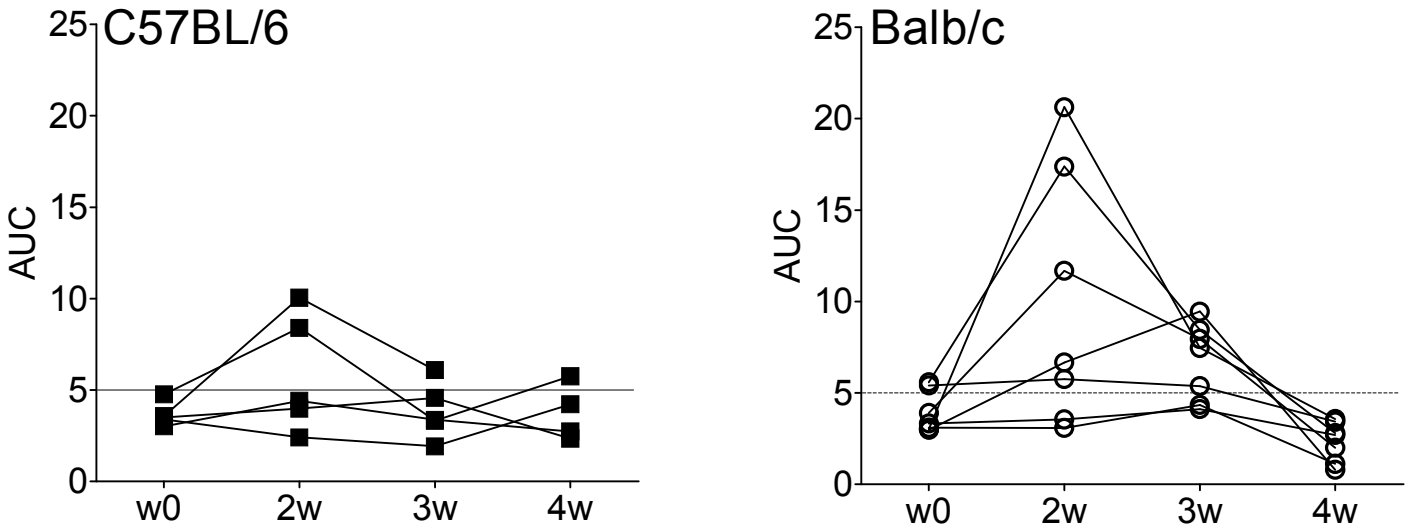

Non-infected
C. rodentium
A
10d
14d
5w
Crypt length (μm)
400
300
200
100
0
p = 0.029
p = 0.0007
p = 0.63
Non-infected
C. rodentium
B
10d
14d
5w
p < 0.0001
p = 0.0064
p = 0.67


A
C57BL/6
B
Balb/c
Pre-infection
Week 2 PI
Week 3 PI
Week 4 PI
Week 5 PI + 24hr post-WAS
VMR relative to maximum pain at pre-infection (%)
Distension volume (µl)
Disension volume (µl)
300
200
100
0
20
40
60
80
*
**
C
D
AUC
25
20
15
10
5
0
p = 0.018
p = 0.061
Pre-inf
2w
3w
4w
5w
+ WAS
PI


A
relative IFN-g expression
10-5
relative TNF-a expression
10-5
relative IL-1b expression
10-4
relative IL-17 expression
10-5
relative IL-6 expression
10-7
relative MCP-1 expression
10-5
relative IL-4 expression
10-5
relative IL-10 expression
10-5
B
relative IFN-g expression
10-5
relative TNF-a expression
10-5
relative IL-1b expression
10-4
relative IL-17 expression
10-5
relative IL-6 expression
10-7
relative MCP-1 expression
10-5
***
relative IL-4 expression
10-5
relative IL-10 expression
10-5
Non-inf
10d
14d PI

Supplementary Fig. 2




C57BL/6
AUC
25
20
15
10
5
0
w0
2w
3w
4w
Balb/c
AUC
25
20
15
10
5
0
w0
2w
3w
4w

Dear Editor,

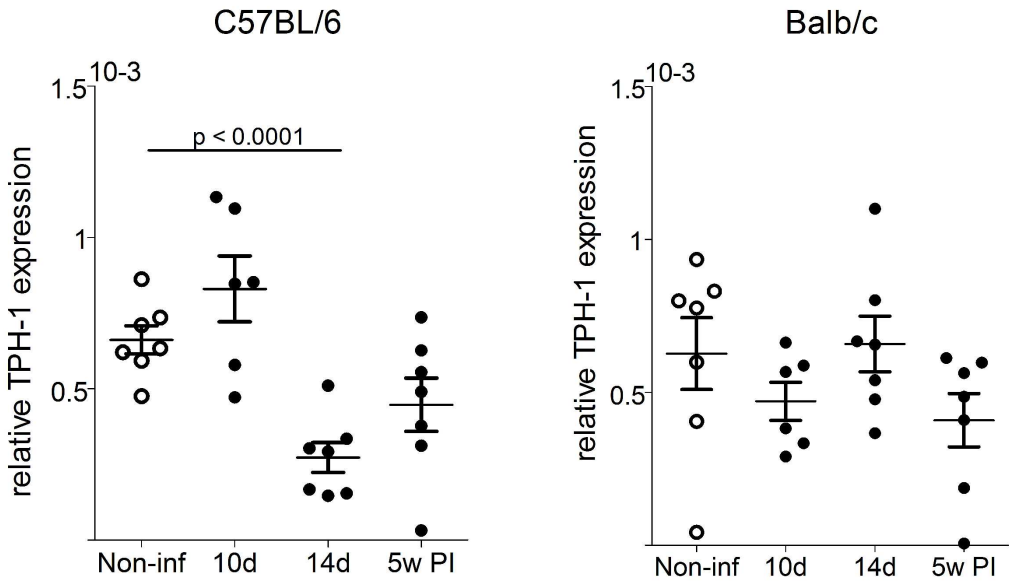
We thank the reviewers for their comments and proposed suggestions. We have addressed all the issues raised and changed the manuscript accordingly. We are convinced that our revised manuscript has improved and hope it will be acceptable for publication in Neurogastro-enterology & Motility.

Yours sincerely,
Mira Wouters
Guy Boeckxstaens

Reviewer 1 Comments:

The authors provide a revised manuscript detailing the strain-dependent differences in visceral hypersensitivity following Citrobacter rodentium infection. The single most important addition to the manuscript is the addition of data demonstrating changes in mast cell tryptase expression in the animals. These data support the importance of mast cells in visceral hypersensitivity, and provide the only evidence of a difference in immune responses that could explain differences in visceral hypersensitivity. The manuscript is greatly improved by this and the other changes. In light of these new data, the discussion could be more dramatically re-written, than what has been done. There remain only minor concerns with the manuscript. Also for the authors' edification in response to their response to reviewer 1 comment #4, murine mast cells (as opposed to humans) contain TPH1 that is important for immune function (Nowak et al., J Exp Med. 2012 209:2127).

Reply: We thank the reviewer for his/her comments. As already indicated in our previous reply, the expression of TPH1 mRNA is very high compared to tryptase a/b (i.e. more than 10 cycles different or 1024 fold increased), suggesting that TPH1 is not only expressed by mast cells but also by enterochromaffin cells, diluting the expression of mast cell-specific TPH1 and making it impossible to pick-up small differences in mast cell-specific TPH1. As proposed by the reviewer, we include here our TPH1 mRNA expression results in colonic tissue at various time points (see figure below). No significant differences for TPH1 mRNA levels were observed between non-infected and infected Balb/c mice, at any time point. However, for C57BL/6 mice, we found a significant decrease in TPH1 expression at 14 days PI compared to non-infected controls. This drop may be due to colonic mucosal damage involving enterochromaffin cells and consequently decreasing TPH1 expression. Of note, the damage observed in C57BL/6 mice is more pronounced (p = 0.0007) than in infected Balb/c mice (p = 0.0064) as shown in figure 3 of the manuscript. As TPH1 mRNA expression is not mast cell specific and the paper already contains many figures, we propose not to include this figure. Hopefully the reviewer can agree with our decision.



Minor concerns:

1. Regarding original major concern #1, that the manuscript relies too heavily on strain dependent differences in immune responses rather than other potential strain-dependent differences, the authors have revised paragraph 3 of the discussion. Besides this paragraph, however, the discussion was not appreciably changed overall and the major conclusion remains that the TH2 predominant background may be causative (see statements below). Given the data, these statements seem misleading to the reader and it is recommended that the overall tone of the discussion and abstract be changed. Of particular interest, paragraph 1 of the discussion states "The increase in visceral nociception was transient and lasted longer in the Th2-predominant Balb/c mice." Paragraph 4 of the discussion states "These data together with our current findings seem to support the hypothesis that the immunogenetic background may, at least to some extent, contribute to the risk to develop PI-IBS." Paragraph 6 of the discussion (conclusion) states "In summary, our study shows that C. rodentium infection induces a transient VHS in C57BL/6 and Balb/c mice, that is more pronounced and prolonged in Th2-predominant Balb/c mice, indicating that a Th2 immune background may increase the susceptibility to develop PIIBS." Finally, the abstract states "Citrobacter rodentium infection induces transient VHS in C57BL/6 and Balb/c mice, which is more pronounced and persisting in Balb/c mice, suggesting that a Th2 background may represent a risk factor for prolonged PI-VHS."

   Reply: *We regret that the reviewer is not satisfied with our previous attempt to weaken our statements on the role of the immunogenetic background. We replaced "indicate" by "suggest" and included "may" in every statement, and provided alternative explanations for the observed difference between C57BL/6 and Balb/c mice. We agree that there are only minor changes in immunological parameters, but even in the food allergy model we routinely use in our lab (where mice sensitized to ovalbumin develop hypothermia, mast cell activation and diarrhea upon ovalbumin gavage), only minor changes can be detected using real time qPCR of intestinal tissue. In other words, it is a misconception to expect large changes in expression of Th2 cytokines.*

Mainly based on our follow-up studies (showing failure of oral tolerance development and prolonged mast-cell mediated VHS in infected Balb/c but not in C57BL/6 mice) in the post-infectious model used in the present manuscript (see reference 65 and 66 in the discussion at p.15), we feel confident that the Th2 background is a major determinant in the development of VHS. These data are however currently extended and cannot be included in the current paper. Nevertheless, we further weakened some of the statements cited above by the reviewer:

1. *Abstract: We added: "Although other strain-related differences may contribute, a Th2 background may represent a risk factor for prolonged PI-VHS." and we deleted the following sentence: "suggesting that a Th2 background may represent a risk factor for prolonged PI-VHS".*
2. *Discussion first paragraph p. 12: We rephrased as follows: "These results suggest that a Th2-predominant immunogenetic background may represent one of the risk factors to develop prolonged abnormal visceral nociception following an episode of infectious gastroenteritis. Of note, other strain-related factors, such as differences in nociception and behavior, may undoubtedly contribute as well." and deleted the following statement: "This VHS is however transient, suggesting that other factors or triggers, resulting in a sustained abnormal pain response as observed in PI-IBS patients, must be involved."*
3. *Discussion last paragraph p.16: We deleted the following sentence: "indicating that a Th2 immune background may increase the susceptibility to develop PI-IBS." and we rephrased the last sentence into: "Although other strain-related differences, such as differences in nociception and behavior, may contribute, our data suggest that a Th2 background may represent an additional risk factor for prolonged PI-VHS. It should be emphasized though that PI-VHS was transient and thus other factors must be involved in the persistent VHS as observed in patients with PI-IBS.*

We hope the reviewer can accept this.

2. The authors stated in response to the original minor concern (#10) that stress was not investigated at the three week time point when strain-dependent differences in hypersensitivity was observed, that "It is unclear why the reviewer would be interested to combine stress at this time point, especially as the aim of the study is to investigate strain-related differences in infection-induced VHS, and not in stress-induced differences." If so, why was stress part of the experimental protocol at all? In the discussion, the authors state that a lack of stress-induced visceral hypersensitivity is not surprising because stress during or before, but not after infection is associated with visceral hypersensitivity. Again, the experiment does not seem to be framed well nor adequately discussed. If stress causes changes in sensitivity only during or before infection, should this rather than PI stage have been studied?

Reply: We completely agree with the reviewer that a different set of experiments should have been performed to study the interaction between stress and infection, including experiments where stress is applied during or before the infection. These experiments have been reported previously by Ibeakama et al. and Spreadbury et al. indeed showing that stress applied before the infection rather than in the post-infectious phase increases the susceptibility to PI-VHS (Ibeakanma et al., 2009; Spreadbury et al., 2015) but this was only assessed in C57BL/6 mice and not in Balb/c mice. However, as the aim of our study was to investigate the role of the genetic

background and not the interaction between stress and infection as such, we hope the reviewer agrees that we decided not to perform these experiments. The only reason to add stress after the infection was to check if a previous infection, similar to maternal separation, could be a factor leading to increased susceptibility to develop stress-induced VHS. If the reviewer feels however we have to exclude these data, we are happy to do so. We already deleted the last sentence of the manuscript: "An acute episode of stress in the post-infectious phase could not re-introduce VHS indicating that other mechanisms leading to persistent VHS, as observed in patients, must be involved."

3. The second paragraph of page 15 remains speculative and outside of the range of the current manuscript. Food allergens are completely outside the aims of the current study and for the authors to state that they are currently working on this hypothesis seems inappropriate. If food allergens are so important, it begs the question why was stress used as a reinitiating factor, which the authors acknowledge is not expected to cause hypersensitivity at the time point they used, rather than food allergens. I would recommend, again, deleting this paragraph.

   Reply: We agree that food allergens are completely outside the aim of the current study. However, in view of the largely negative findings on persistent or chronic VHS, we feel that speculation on potentially other explanations is part of an interesting discussion, and is of great interest for the reader to better position our data. We would therefore propose that the editor decides on keeping the paragraph about the food antigens in the discussion or not.

4. There is no discussion regarding the apparent difference of no observable visceral hypersensitivity at 4 weeks in the current study and previous studies using C57Bl6 mice demonstrating enhance hyperexcitability of nociceptive DRG neurons at 30 days post-infection (Ibeakanma et al.).

   Reply: We thank the reviewer for this comment. We have implemented this in the discussion in the first paragraph of p.14: "It should also be emphasized though that the VHS observed in both strains completely normalized during the PI phase (i.e. at 4 weeks PI), while patients with PI-IBS continue to have symptoms for several years following the infectious episode. These findings are in accordance with other studies, showing no increased VMR response in C57BL/6 mice 30 days PI[22]. Hence, other mechanisms may be critical for the development of chronic VHS."

Reviewer 2 Comments:

Just one further grammatical comment page 15 line 22: Therefore, we checked if WAS could install prolonged VHS when applied in the post-infectious period. In the present study however, acute WAS at 5 weeks PI did not re-install VHS, irrespective of genetic background. "install" is a curious use of the word. I suggest: "Therefore, we checked if WAS could cause prolonged VHS when applied in the post-infectious period. In the present study however, acute WAS at 5 weeks PI did not recreate VHS, irrespective of genetic background".

<u>Reply:</u> We have rephrased the first sentence and adjusted the comment as suggested (discussion p.14, 2nd paragraph): "Therefore, we evaluated if a previous gastrointestinal infection would increase the risk to develop VHS in response to WAS. In the present study however, acute WAS at 5 weeks PI did not recreate VHS, irrespective of genetic background".